TITLE: PURIFIED   PLANT   EXPANSIN   PROTEINS   AND   DNA
ENCODING SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

5   This specification is a continuation-in-part of U.S. Application Serial No
09/896,301, filed June 29, 2001; which is a Continuation of U.S. Application Serial
No. 09/092,160 filed June 5, 1998, now U.S. Patent No. 6,255,466, issued July 3,
2001; which is a Divisional of U.S. Application Serial No. 09/429,675 filed
October 28, 1999, now U.S. Patent No. 6,326,470, issued December 4, 2001; which

10   is a Continuation-in-part of U.S. Application Serial No. 08/834,327 filed April 15,
1997, now U.S. Patent No. 5,990,283, issued November 23, 1999; which is a
Continuation-in-part of U.S. Application Serial No. 08/444,515 filed May 12, 1995,
now U.S. Patent No. 5,959,082, issued September 28, 1999; which is a
Continuation-in-part of U.S. Application Serial No. 08/242,090 filed May 12, 1994

15   now abandoned; which is a Continuation-in-part of U.S. Application Serial No.
08/060,944 filed May 12, 1993, now abandoned.

BACKGROUND OF THE INVENTION

          The present invention relates to a new class of proteins, known as

20   expansins, and their isolation, sequencing, genesis by expression systems, and
utilization. These proteins have been identified in a wide variety of plant and other
materials and have a variety of applications, including but not limited to
agricultural and/or food applications and industrial uses such as their use in the
paper industry as a catalyst for weakening the strength of paper products. For

25   example, they can prove especially useful in the recycling of paper.

          By the way of background, for many years wall "loosening enzymes" have
been implicated in the control of plant cell enlargement (growth), largely on the
basis of rapid biophysical and biochemical changes in the wall during auxin-
induced growth (reviewed by Cleland and Rayle, Bot. Mag. Tokyo, 1:125-139,

30   1978; Taiz, *Annu. Rev. Plant Physiol.,* 35:585-657, 1984). Plant walls contain
numerous hydrolytic enzymes, which have been viewed as catalysts capable of

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weakening the wall to permit turgor-driven expansion (reviewed by Fry, *Physiol. Plantarum*, 75:532-536, 1989). In support of this hypothesis, Huber and Nevins (*Physiol. Plant.*, 53:533-539, 1981) and Inoue and Nevins (*Plant Physiol.*, 96:426-431, 1991) found that antibodies raised against wall proteins could inhibit both auxin-induced growth and wall autolysis of corn coleoptiles. In addition, isolated walls from many species extend irreversibly when placed under tension in acid conditions in a manner consistent with an enzyme-mediated process (Cosgrove D.J. *Planta*, 177:121-130, 1989). Despite these results and other evidence in favor of "wall-loosening" enzymes, a crucial prediction of this hypothesis has never been demonstrated, namely, that exogenously added enzymes or enzyme mixtures can induce extension of isolated walls. To the contrary, Ruesink *(Planta.* 89:95-107, 1969) reported that exogenous wall hydrolytic enzymes could mechanically weaken the wall without stimulating expansion. Similarly, autolysis of walls during fruit ripening does not lead to cell expansion. Thus a major piece of evidence in favor of wall-loosening enzymes as agents of growth control has been lacking.

Once identified, expansins--proteins capable of inducing cell wall extension--would have utility not only in the engineered extension of cell walls in living plants but foreseeably in commercial applications where their chemical reactivity could prove useful. If expansins can disrupt noncovalent associations of cellulose, as they have been shown to do, then they would have particular utility in the paper recycling industry. Paper recycling is a growing concern and will prove more important as the nation's landfill sites become more scarce and more expensive. Paper derives its mechanical strength from hydrogen bonding between paper fibers, which are composed primarily of cellulose. During paper recycling, the hydrogen bonding between paper fibers is disrupted by chemical and mechanical means prior to re-forming new paper products. Proteins which cause cell expansion are thus intrinsically well suited to paper recycling, especially when the proteins are nontoxic and otherwise innocuous, and when the proteins can break down paper products which are resistant to other chemical and enzymatic means of degradation. Use of proteins of this type could thus expand the range of recyclable papers.

Other modes of application of expansins, once they are found, include production of virgin paper. Pulp for virgin paper is made by disrupting the bonding between plant fibers. For the reasons identified above, expansins are useful in the production of paper pulp from plant tissues. Use of expansins can substitute for

5   harsher chemicals now in use and thereby reduce the financial and environmental costs associated with disposing of these harsh chemicals. The use of expansins can also result in higher quality plant fibers because they would be less degraded than fibers currently obtained by harsher treatments.

Thus, a need remains for the identification, characterization and purification

10   of expansins--proteins which can be characterized as catalysts of the extension of plant cell walls and the weakening of the hydrogen bonds in the pure cellulose paper--and the incorporation of DNA sequences which give rise to such proteins in appropriate expression systems.

15   BRIEF SUMMARY OF THE INVENTION

In accordance with the present invention, a new class of proteins and methods related thereto are presented. The proteins, which can be characterized as catalysts of the extension of plant cell walls and the weakening of the hydrogen bonds in pure cellulose, are referred to as expansins. Two proteins have been

20   isolated by fractionation techniques from washed wall fragments of cucumber hypocotyls, referred to as "cucumber expansin-29" and "cucumber expansin-30" (abbreviated cEx-29 and cEx-30). Moreover, three peptide fragments from the purified cEx-29 protein were sequenced, then oligonucleotide primers of about 4-30 contiguous nucleotides were designed to amplify a portion of the expansin cDNA

25   using polymerase chain reaction with a cDNA template derived from cucumber seedlings, and then the PCR fragment was used to screen a cDNA library to identify full length clones. Another expansin protein has been isolated from oat coleoptiles (oat expansin oEx-29), while three additional expansin sequences have been identified in *Arabidopsis* and an additional two in rice. Expansins appear to be

30   broadly distributed throughout the plant kingdom and can be identified in stem and leaf vegetables (i.e., broccoli, cabbage), fruit and seed vegetables (i.e., tomato),

fiber crops and cereals (i.e., corn), and forest and ornamental crops (i.e., cotton). Also, expansin-like protein has been found in proteins obtained from the digestive tract of snail and its feces (sEx). These novel proteins can find use in a variety of applications including the paper industry in production of paper pulp in preparation

5   of virgin paper and in paper recycling as a preferred way of disruption of paper fibers due to their nontoxic and environmentally innocuous nature in contrast with the harsh chemical treatments applied today, which are environmentally noxious and in many cases completely unacceptable.

10   BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Extension curves of native and reconstituted isolated cell wall specimens obtained using a constant load extensometer.

Ten-mm sections of tissue were frozen and thawed, and lightly abraded prior to suspension in the extensometer. Tissues were clamped under an applied

15   force of 20g and extension recorded using a linear voltage displacement transducer. The length of tissue between the clamps was 5 mm.

(A)   Extension curves of native and reconstituted cucumber hypocotyl walls. From top to bottom: native walls were suspended under tension in 50 mM Hepes, pH 6.8, for 20 minutes after which the bathing solution was replaced by 50

20   mM "sodium acetate, pH 4.5, (arrow). Inactivated walls were treated for 15 sec with boiling water prior to suspension; as shown in the second line, this treatment eliminated acid induced extension. For reconstitution experiments, inactivated walls were suspended in 50 mM sodium acetate, pH 4.5, for 30 minutes at which point the bathing solution was replaced by 0.5 mL of fresh solution (arrows)

25   containing 2-3 mg of proteins extracted from growing cell walls (apical wall protein), or with soluble proteins from growing cells (apical soluble proteins), or proteins extracted from cell walls from the nongrowing cotyledon (cotyl. wall protein) or from walls of the basal hypocotyl (basal wall protein).

(B)   Reconstitution of extension activity in inactivated (heat treated)

30   walls from growing tissues of different species by the active cucumber wall extract. Walls from the growing region from tomato, pea and radish hypocotyls, lily and

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onion leaves, and coleoptiles of maize and barley were prepared, heat-treated, and suspended as given above, except that a force of only 10g was applied to the more fragile tomato and radish walls. Walls were first suspended in 50 mM sodium acetate, pH 4.5, after 30 minutes (arrow) the bathing solution was replaced with 0.5 mL of the same buffer containing 2-3 mg of active wall protein from cucumber hypocotyls (apical 3 cm).

Figure 2. Fractionation of extension-inducing cucumber wall extracts by HPLC.

(A)   Ammonium sulfate precipitates of salt extracted cucumber wall extracts were resuspended and loaded onto a C3"hydrophobic interactions column in 50 mM sodium acetate, pH 4.5, 20% (saturation) ammonium sulfate. Proteins were eluted in a descending gradient (20-0%) of ammonium sulfate. Fractions were desalted and checked for extension inducing activity with inactivated cucumber tissues as in Figure 1. A_{280} nm is shown by a solid line and extension inducing activity by a broken line.

(B)   Active fractions from (A) were concentrated and desalted into 15 mM Mes, pH 6.5, and loaded onto a sulfopropyl anion exchange column equilibrated with the same buffer. Proteins were eluted with an ascending gradient of NaCl (0-1M) in this buffer. Extension inducing activity (broken line) of fractions was checked directly after adjusting the pH to 4.5 with 1M acetic acid.

Figure 3. SDS-PAGE of ammonium sulfate precipitate (AS), active fractions from C3 and sulfopropyl (S1 and S2) HPLC separations of cucumber proteins.
Protein samples were concentrated, desalted, and run on SDS-polyacrylamide gels according to the method Laemmli. Gels were stained with Coomassie Brilliant Blue R 250. Arrows indicate the major protein bands at 29 and 30 kD in the S1 and S2 fractions which appear to possess the extension-inducing activity.

Figure 4. The effects of DTT, metal ions, methanol and water boiling, and protease treatments on reconstituted extension.

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(A) The effect of DTT on reconstituted extension. Growing wall specimens of cucumber hypocotyl were inactivated by heat and then reconstituted by shaking in a solution of active C3 proteins (estimated concentration of 50 micrograms per mL). Walls were then clamped under constant load (as described in Figure 1) firstly in a bathing solution of 50 mM Hepes, pH 6.8. After 20 minutes the solution was changed for 50 mM sodium acetate, pH 4.5 (first arrow). After a further 40 minutes DTT (from a 100 mM stock solution) was added to give a final concentration of 10 mM. The first two lines represent typical traces from 4 experiments with and without DTT addition.

For the effects of metal ions, growing wall specimens from cucumber hypocotyl were inactivated by heat and then clamped under constant load (as described in figure 1) in a bathing solution of 50 mM sodium acetate, pH 4.5. After 20 minutes the bathing solution was exchanged for a fresh one containing 50 micrograms per mL of active C3 proteins (first arrow). After a further 40 minutes $AlCl_3.6H_2O$ or $CuCl_2.2H_2O$ (from 100 mM stock solutions) was added to bring the bathing solution to a final concentration of 1 mM (second arrow). All experiments were repeated 4 times. Line 3 shows extension without the addition of metal ions, the next two lines show typical data obtained with the addition of $AlCl_3$ and $CuCl_2$ respectively.

For the effects of boiling cell walls in methanol or boiling in water on the recovery of extension inducing activity, growing cucumber hypocotyls tissue was first boiled for 3 minutes in methanol or for 30 sec. in distilled water, fragments were recovered, cleaned and extracted (as described in Figure 1). Proteins were precipitated with $(NH_4)_2SO_4$ and resuspended in 50mN sodium acetate, pH 4.5 before being tested in the reconstitution assay described in Figure 1. Lines 6 and 7 are representative data from 4 experiments.

(B) Growing wall specimens were inactivated by heat and reconstituted with C3 proteins. Reconstituted walls were incubated with 1000 units of trypsin or chymotrypsin for 4 hr. at 30°C, in 50 mM Hepes, pH 7.3, or with 2 milligrams per mL of pronase or papain for 4 hr. at 30°C, in 50 mM sodium acetate, pH 5.0. Controls were reconstituted and incubated in the same manner without the addition

of proteases. At the end of the incubation tissues were clamped under constant load (as described in Figure 1), first in 50 mM Hepes, pH 6.8. After 30 minutes the bathing solution was replaced by 50 mM sodium acetate, pH 4.5. The difference in the two rates of extension was calculated. Data presented are the means of four experiments in each case.

Figure 5. Effect of pH on reconstituted extension.

Cucumber wall specimens, inactivated by heat, were clamped under constant load (as described in Figure 1). Initial bathing solutions were 50 mM citric acid, titrated to pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 or 6.5 with 1 M K_2HPO_4. After 30 minutes, the bathing solution was changed for a 1:1 dilution of active C3 proteins with the relevant buffer (final estimated protein concentration was 50 micrograms per mL), where necessary the final pH was adjusted using either 1 M citric acid or 1 M K_2HPO_4. Extension was recorded for a further 2 hr and change in rate of extension calculated as initial - final rates.

Figure 6. The effects of cucumber expansins on cellulose paper.

(A)   Strips of Whatman No. 3 filter paper were clamped in a constant load extensometer with 5 mm of paper between the clamps (described in Fig. 1). Paper strips were initially bathed in 50 mM sodium acetate, pH 4.5. After about 20 minutes of extension, the bathing solution was replaced with either: 5 mg of S1 protein in 15 mM Mes, pH adjusted to 4.5 with 1 M acetic acid (S1); 5 mg of S1 protein, in the same buffer, which had been heated to 100°C for two minutes (Boiled S1); 100 mg of cellulase (from Trichoderma viride Boehringer Mannheim) in 50 mM sodium acetate (Cellulase), pH 4.5; or 50 mM sodium acetate pH 4.5 (Control). Extensions were recorded for a further 100 minutes. The figure shows representative traces from at least six independent experiments, all of which showed similar results.

(B)   Strips of Whatman No. 3 paper were soaked in a solution containing 5 mg/mL S1 protein in 15 mM Mes, pH adjusted to 4.5 with 1M acetic acid, (S1); in the same protein solution which had been boiled for two minutes (Boiled S1); or

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in 50 mM sodium acetate, pH 4.5 (Control). Stress relaxation tests were performed by standard methods. Data represent the average of ten measurements in each case. Experiments were repeated three times with similar results.

(C)   Conditions were the same as in (B) except that the paper strips were soaked in a solution containing 100 mg/mL of Trichoderma cellulase in 50 mM sodium acetate, pH 4.5 (Cellulase), or 50 mM sodium acetate, pH 4.5 (Control). Data are the averages of ten measurements in both cases. Experiments were repeated three times with similar results.

Figure 7. The effect of cucumber expansins on slick paper.

Paper strips of 2-3 mm width were cut and hung in our extensometer, such that a 5-mm long strip was put under 20-g force. The buffer contained 50 mM sodium acetate, pH 4.5 with or without added C3 protein (about 20 pg per paper strip was used). The length of the paper strips was recorded for up to 5 hours. Four samples of each paper were used with each treatment (+/- protein). The untreated (control) paper showed very little change in length, and maintained its mechanical strength throughout the test period. In contrast, the treated papers began to extend and quickly broke once extension began. The time for breakage after addition of protein varied from 45 minutes to 2.5 hours, but all samples broke.

Figure 8. Acid-induced extension in native and heat-treated oat coleoptile walls.

(A)   When switched from a neutral buffer (50 mM Hepes, pH 6.8) to-an acidic-buffer (50 mM sodium acetate, pH 4.5), native walls extended at a high rate which fell continuously with time, whereas heat-treated walls lacked a significant response. The native and heat-treated (15 sec in boiling water) walls were prepared and mounted on an extensometer (5 mm between two clamps) with 20 g of tension as described by Cosgrove (1989) and McQueen-Mason et al. (1992) .

(B)   A plot of extension rate for the native walls shown in A demonstrates that the rate decreases continuously. Similar results were obtained in 10 repetitions.

Figure 9. Effect of dithiothreitol (DTT) addition to acid-induced extension of oat coleoptile walls.

Native walls were prepared as in Figure 8 and the buffer was exchanged for the same buffer, containing 10 mM DTT at approximately 80 minutes after start of acid-induced extension.

(A)   The plot of extension rate (B) shows that the rate is stable at about 5% per hour after addition of DTT. Similar results were obtained in 6 independent trials.

Figure 10. Acid-induced extension in native oat coleoptile walls in the continuous presence of DTT.

(A)   Walls were prepared as in Figure 8 and incubated in 50 -mM Hepes, pH 6.8, with 2 mM DTT; after about 35 minutes the buffer was switched to 50 mM sodium acetate, pH 4.5, with 2 mM DTT.

(B)   The plot of extension rate shows that the decay in extension rate after 1 hour is greatly diminished (compare with Figure 8). This experiment was repeated four times with similar results.

Figure 11. Extension induced in oat, cucumber and pea walls by proteins extracted from oat coleoptiles walls (A) and cucumber walls (13).

Coleoptile wall proteins and cucumber wall proteins were extracted with 1 M NaCl, precipitated with ammonium sulfate, desalted and partially purified on a DEAE Sephadex column. Heat-inactivated cell walls from oat coleoptiles, cucumber hypocotyls or pea epicotyls were clamped at 20-g tension in 50 mM sodium acetate, pH 4.5. After about 0.5 hour, the solution was replaced with 0.4 mL of the same buffer containing about 10 mg proteins partially purified from oat or cucumber cell walls by DEAE-anion exchange chromatography. The data represent a typical experiment from at least four replicates.

Figure 12. Comparative responsiveness of oat and cucumber walls to exogenously added oat or cucumber proteins.

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(A)   Concentration dependence of cucumber and oat walls to exogenous cucumber protein. Cucumber walls were frozen, thawed, abraded, heat-inactivated and loaded in the extensometer, as in Figure 8.   Oat coleoptiles were treated similarly, except that the epidermis was stripped with fine forceps and the coleoptile bisected longitudinally prior to -freezing. The walls were extended for approximately 30 minutes in 50 mM sodium acetate, pH 4.5, before the buffer was exchanged for the same one containing various concentrations of partially purified cucumber expansins (the active fraction from the C3 HPLC separation). Extension activity is calculated as the increase in extension rate in the first 15-20 minutes after addition of C3 protein and is expressed as % increase in length per hour. The average extension rate prior to addition of protein in these experiments was 2% per hour for oat walls and 1.7% per hour for cucumber walls.

(B)   Response of oat and cucumber walls to added oat protein. Heat-inactivated oat and cucumber walls were prepared as in (A) and extended in sodium acetate, pH 4.5, for about 30 minutes prior to exchange of buffer for the same one containing 73 μg of crude oat coleoptile protein (ammonium sulfate precipitate) per sample holder (about 400 μL). The extension curves shown illustrate two examples with responses close to the mean (2.8% h-^{1} increase for the cucumber wall and 1.1% h-^{1} increase for the oat wall). The mean responses (n=4) of oat and cucumber walls to added oat protein are shown in the inset and are calculated as the increase in extension rate, expressed as % per hour. The baseline extension rates prior to addition of protein averaged 2.4 % per hour (oats) and 1.1% per hour (cucumber).

Figure 13. Effect of 1 M NaCl extraction on acid-induced extension of oat coleoptile walls.

Coleoptiles were abraded prior to freezing, then either directly clamped, in the extensometer or extracted for 16 hours in 1 M NaCl containing 10 mM Hepes, pH 6.8, 3 mM EDTA and 2 mM sodium bisulfite prior to measurement. Walls were rinsed briefly in water, clamped at 20g tension, then incubated in 50 mM Hepes, pH 6.8, with 2 mM DTT. At about 30 minutes the buffer was exchanged for 50 mM sodium acetate, pH 4.5, with 2 mM DTT. The curves shown are

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representative of eight trials for each treatment. The inset shows the average extension response (SE, n=8) of each treatment. Response is calculated as the rate at 1.5 to 2 hours minus the rate prior to pH 4.5 buffer, and is expressed as % increase in length per hour.

Figure 14. pH dependence of the extension activity reconstituted by oat wall proteins.

Heat-inactivated walls from cucumber hypocotyls were clamped in an extensometer as in Fig. 8 and placed in 50 mM sodium acetate buffers at pH 3.5, 4.0, 4.5, 5.0 or 5.5. After 20 minutes, the solutions were replaced with 0.4 mL of the corresponding buffer containing 10 mg oat-coleoptile wall proteins partially purified by DEAE-chromatography. The extension activity was calculated by subtracting the baseline rate without proteins from the linear rate (5-30 minutes) after the addition of the proteins, and expressed as % increase in length per hour above the baseline rate. The data represent the means ± S.E. (n=5 to 8). The average baseline rate prior to addition of protein ranged between 2.52 and 2.96 μm min^{-1} (or 3.0 to 3.55 % per hour) for all pH groups.

Figure 15. Purification of oat expansin by high pressure liquid chromatography (HPLC) on a carboxymethyl (CM) cation exchange column.

(A)   Elution of proteins (absorbance at 280 nm) from a CM-column. Proteins were solubilized from the cell walls of etiolated coleoptiles by 1 M NaCl and then sequentially fractionated by ammonium sulfate precipitation, DEAE-chromatography, and ConA chromatography, prior to CM-HPLC.

(B)   Wall extension activity of HPLC fractions. The extension activity was assayed by addition of fraction samples (equal volumes, typically 20 μL) to sample cuvettes containing 400 /μL of 50 mM sodium acetate buffer, pH 4.5. Activity is expressed as increase in extension rate after addition of protein to a 5-mm, heat inactivated, abraded oat coleoptile. A single peak of activity eluted at about 15 minutes or 3.5% of 1 M NaCl. Similar results were obtained in six independent trials.

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Figure 16. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins from different purification steps.

Active protein fractions were separated on a SDS-PAGE gradient gel (4-20%) and stained with Coomassie Blue R-250. Lane 1, 1 M NaCl extraction (20 mg) ; lane 2, ammonium sulfate precipitate (20 mg); lane 3, active DEAE-fraction (10 mg); lane 4, proteins passing through Con A column (5 mg) ; lane 5, active fraction from CM-HPLC (0.3mg). The protein with apparent molecular mass (by SDS-PAGE) of about 29 kD was designated as oat expansin (oat Ex29). Similar results were obtained in - five trials, except in some of these an additional protein band at about 35 kD appeared in lane 5.

Figure 17. Western analysis of oat coleoptile proteins probed with antiserum against cucumber Ex29.

Comparison of lane I (crude wall protein) with lane 2 (crude protoplasmic protein) shows that the antiserum recognizes an Ex29-like protein (arrow) specifically bound to the coleoptile cell wall. Comparison of lane 3 (crude wall protein) with lane 4 (purified protein with extension activity) shows that the Ex29-like protein copurifies with the extension activity. Methods: Lane 1 was loaded with 15 μg of crude wall protein (ammonium sulfate precipitate of 1 M NaCl extract). Lane 2 had 15 μg of the soluble protoplasmic protein fraction. These samples were separated on the same-4-20% gradient SDS polyacrylamide gel, blotted onto nitrocellulose, and probed with rabbit antiserum against cucumber Ex29. Lane 3 was loaded with 0.2 μg of crude wall protein and Lane 4 had 0.2 μg of active oat wall-extension protein purified sequentially by DEAE Sephadex and CM-HPLC. These samples (3-4) were separated on the same 14% SDS polyacrylamide gel, blotted onto nitrocellulose, and probed with antiserum. This assay, with minor variations, was carried out four times with similar results.

Figure 18. Extension induced in isolated cucumber walls by proteins from snails.

Cucumber walls were prepared as in the description for Fig. 11. Proteins prepared by dissolving snail acetone powder (10 mg/mL) in 50 mM sodium acetate, pH 4.5, are capable of inducing extension of isolated cucumber walls. The

immediate response and the linearity of the extension are unique characteristics that indicate an expansin-like protein may be involved.

Figure 19. Extension induced in isolated cucumber walls by proteins from snails' feces.

The feces of Helix aspersa (snail) induced extension in cucumber walls. The feces were dissolved in 50 mM sodium acetate buffer, pH 4.5. The procedure was as described in Figure 11.

Figure 20. Western blot analysis of active HPLC-separated proteins fraction from snail.

Acetone powder solution was proved with antibody which was raised against cucumber expansin -29. The analysis was performed as described in Figure 17. The active fractions show a striking band at about 26 kD, which is similar to cucumber expansin-29. These results provide strong evidence that the wall extension activity found in the snail acetone powder is due to a protein of similar size and similar antigenic determinants as cucumber expansin-29.

Figure 21. Western blot of the protein from Helix aspersa feces, probed with the antibody PA-1.

Feces from Helix aspersa were dissolved as described in Figure 19 and Western blot analysis was performed in Figure 20. The single band indicates the presence of a protein antigenically similar to cucumber expansin-29.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention describes the first evidence of a new class of proteins named expansins, as well as their purification, sequencing, expression and use. During the course of detailed studies of plant cell growth, our laboratory isolated first from cucumber two novel proteins which we named expansins. Further research found expansins in *Arabidopsis*, rice and oat coleoptile as well as in the proteins obtained from the digestive tract of snails. An important aspect-of

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expansin technology is the identification of the defining characteristics of the expansin protein at a molecular level, and the incorporation of complementary gene sequences into appropriate expression systems for large-scale manufacture of the desired expansin proteins.

5   Several detailed embodiments of the present invention are disclosed herein, including cucumber, *Arabidopsis*, rice and oat proteins, as well as an expansin-like protein from snail digestive tract. However it should be understood that these embodiments are merely illustrative of the invention, which may be embodied in various forms and with predictable divergence of amino acid sequence within the

10   class of expansins described herein. Generally speaking, a particular amino acid sequence (SEQ ID NO:7), or greater than about 60% sequence similarity and preferably greater than about 70% sequence similarity with that sequence, characterizes the individual members of the class of purified proteins which constitute the inventive expansin genus. Ultimately, however, the dispositive factor

15   in defining an expansin is whether it behaves as an expansin, or exhibits the characteristics of an expansin, as discussed throughout this specification and the parent applications identified above.

We herein present evidence that the walls of growing cucumber seedlings possess extractable proteins which can induce extension of isolated walls.

20   Additionally, we identify proteins having this activity and provide the relevant amino acid sequence. We propose the name "expansin" for this class of proteins, defined as endogenous cell wall proteins which restore extension activity to inactivated walls held under tensions. We further propose the specific names "expansin-29" and "expansin-30 (abbreviated Ex-29 and Ex-30, with-respect to

25   their relative molecular masses apparent from SDS PAGE; McQueen-Mason et al. *Plant Cell*, 4:1425-1433, 1992) for the two proteins isolated from cucumber. Moreover, three peptide fragments from the purified cEx-29 protein were sequenced, then oligonucleotide primers were designed to amplify a portion of the expansin cDNA using polymerase chain reaction with a cDNA template derived

30   from cucumber seedlings, and then the PCR fragment was used to screen a cDNA library to identify full length clones. The term "oligonucleotide" refers to a nucleic

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acid molecule generally comprising a length of 200 bases or fewer. The term often refers to single-stranded deoxyribonucleotides, but it can refer as well to single- or double-stranded ribonucleotides, RNA:DNA hybrids and double-stranded DNAs, among others. Preferably, oligonucleotides are 10 to 60 bases in length and most

5   preferably 12, 13, 14, 15, 16, 17, 18, 19 or 20 bases in length. Other preferred oligonucleotides are 25, 30, 35, 40, 45, 50, 55 or 60 bases in length. Oligonucleotides may be single-stranded, e.g., for use as probes or primers, or may be double-stranded, e.g., for use in the construction of a mutant gene. Oligonucleotides of the invention can be either sense or antisense oligonucleotides.

10   An oligonucleotide can be derivatized or modified as discussed above for nucleic acid molecules. The probe is selected to bind to the target nucleic acid, namely the nucleic acid molecule containing the target nucleotide sequence. The oligonucleotide is conveniently sufficiently large to provide appropriate hybridization to the target nucleic acid, yet still reasonably short in order to avoid

15   unnecessary chemical synthesis. Methods for oligonucleotide production are standard in the art.

We have also identified an oat coleoptile wall protein that induced extension in isolated dicot walls (Z.C. Lee et al., 1993, *Planta* 191:349-356). The oat protein has an apparent molecular mass of 29 kD as revealed by SDS-PAGE. For clarity

20   we will refer to the cucumber proteins as cEx, and to the oat proteins as oEx. New data demonstrate that an expansin-like protein may be found in proteins obtained from the digestive tract of snail and its feces. We refer to the snail protein as sEx.

During studies of the biochemical mechanism of action of expansins, we found that they had the ability to weaken the hydrogen bonding between plant cell

25   wall polysaccharides (such as cellulose fibers). These findings allow us to propose the following commercial uses of these novel proteins, including paper treatment, agricultural uses, and a variety of use in food and industrial markets.

The following abbreviations have been used in the specification: ConA = Concanavalin A; CM = carboxymethyl; DEAE = diethylaminoethyl; Hepes

30   = 4- (2-hydroxyethyl)-I-piperazineethanesulfonic acid; DTT = dithiothreitol; Ex29 = 29-kD expansin; Mes = 2-(N-morpholino) ethanesulphonic acid; HPLC = high-

pressure liquid chromatography; PBST = phosphate buffered saline with Tween-20; and SDSPAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis, along with the standard abbreviations for amino acids incorporated in amino acid sequences.

5   Our basic approach was to solubilize and to purify ionically bound proteins from growing cell walls and assay their ability to restore the endogenous extension activity in heat-inactivated walls. Extension was assayed using a constant load extensometer, in which tissue samples were damped under constant force and their extension recorded using an electronic displacement transducer (Cosgrove, D.J.

10 *Planta*, 177:121-130, 1989). The first two proteins possessing the ability to restore the extension activity in heat-in activated wall were solubilized from wall fragments isolated from the growing hypocotyls of dark-grown cucumber seedlings. After various unsuccessful attempts at reconstitution, we obtained a crude salt-solubilized fraction with the ability to induce extension in inactivated walls, shown in Figure

15 1A. The extension induced by this extract mimicked the native extension activity in magnitude and kinetics, i.e., initially high rates decayed over a period of 2 hours to more stable rates of about 2% per hour. These rates are lower than elongation rates of the living stem, but the stress applied to the isolated walls was only one-fifth of the equivalent longitudinal stress imposed on the walls by cell turgor. Like

20 the endogenous extension activity of isolated cell walls, the reconstituted activity required an acid pH and was irreversible (i.e. upon removal of load, the segments did not return to their original length). The addition of protein to native cell walls did not substantially enhance extension, suggesting that endogenous activity was saturating or that binding sites were not accessible to the added material.

25   The active material appeared to be restricted to the growing region of the hypocotyl. When wall fragments from basal (non growing) stem tissue or from the cotyledons (which expand negligibly in our conditions) were extracted, proteins solubilized and purified in the same way did not induce wall extension (Figure IA). These results suggest that non-growing tissues lack the active material; however,

30 we cannot exclude the possibility that the active material was present in the tissue but more firmly bound, inactivated during extraction, or lost during wall isolation.

Soluble cytoplasmic proteins (collected as expressed cell sap or as homogenate) showed no activity, while proteins which were salt solubilized in an equal volume from cleaned wall residues taken from the same tissue showed clear extension inducing activity, indicating an association of the extension activity with the wall.

5   The wall extract from growing cucumber walls did not induce extension of walls from the basal (non-elongating) stem (Figure 1A). Evidently, during maturation the wall is biochemically modified so that it is not susceptible to extension by this material. Perhaps peroxidative cross-linking of lignin or structural proteins such as extension is involved in this loss of sensitivity.

10   The extension activity showed an interesting pattern of species specificity. Figure IB shows that the cucumber wall extract was active on various dicot seedlings (pea, radish, cucumber and tomato) and on monocots of the Amaryllidacea (onion and zephyr lily). In contrast, the extract had a much smaller effect on the coleoptile wall of graminaceous monocots (maize and barley). As

15   graminaceous monocot cell walls differ from those of dicots by having less pectin and-hydroxyproline-rich glycoprotein, and also by having a different type of hemicellulose, it may be that the active fraction extracted from cucumber walls interacts with one or more of these components to induce extension. It may also be that these grass cell walls are cross-linked (not necessarily covalently) in a manner

20   that renders them immune to the cucumber extract. In contrast monocots of the Amaryllidaceae have a cell wall composition more similar to dicots than to the graminae, and this probably explains their susceptibility to the cucumber derived activity.

           The cucumber wall extract was separated by ammonium sulfate

25   precipitation followed by sequential HPLC, shown in Figure 2, first using a hydrophobic interactions column, where a single peak of activity dissipated as the C3 proteins was obtained, and then using a cation exchange column from which the activity was eluted as two distinct peaks. These fractions we have designated S1 and S2 with respect to their order of elution. Figure 3 shows the analysis of the

30   active fractions by SDSPAGE which revealed a major band of relative molecular mass 29 kD associated with S1, while S2 contained a major band at 30 kD (Figure

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3). Active extracts have also been separated by native PAGE and by liquid chromatography with hydroxyapatite, gel filtration media, and DEAE anion exchangers, where activity was consistently associated with these two bands (data not shown). The S1 fraction required only 0.3-1.0 micrograms "of protein to reconstitute extension rates similar to that of native extension, while it required 1.0-2.0 micrograms of S2 to do this.

Cucumber expansins induced extension of walls from several dicot and monocot species, but had little effect when assayed with coleoptile walls from maize and barley. Grass coleoptiles have been a favored object of growth studies since Darwin's analysis of phototropism and have been instrumental in many discoveries about plant growth, including the discovery of auxin. The wall composition of grass coleoptiles is notably different from that of dicots and other monocots nevertheless, coleoptile walls, like dicot walls, do exhibit a strong acid-induced extension *in vitro* and *in vivo*. These observations led us to postulate that a wall protein, with functions-analogous to the cucumber expansins, might mediate the endogenous acid-induced extension of coleoptile walls. Therefore, in further study we attempted to identify oat coleoptile proteins that could induce wall extension, using the wall extraction and reconstitution approach that proved successful with cucumber walls.

To identify the hypothetical oat proteins responsible for wall extension, we used 1 M NaCl to extract proteins from cell walls of etiolated oat coleoptiles. Proteins in this crude extract were precipitated with ammonium sulfate, desalted, and fractionated on a DEAE Sephadex anion exchange column. The unbound proteins passing through this column possessed the ability to induce extension in heat inactivated walls from oat coleoptiles (Figure 11A). Moreover, this active fraction also induced extension of-heat-inactivated cucumber hypocotyl walls and pea epicotyl walls (Figure 11A). This result was -surprising because earlier results led us to expect poor cross-reactivity between extension proteins and walls from dicots and grass coleoptiles.

Figure 8 shows an example of acid-induced extension of oat coleoptile walls. When native walls were clamped under constant tension at neutral pH, the

extension rate was low 30 minutes after application of the load and could be greatly increased by replacing the neutral buffer with a buffer of pH 4.5. The wall extension rate decreased continuously with time after the change to acid buffer (Figure 8B). Addition of 10 mM dithiothreitol, a sulfhydryl reducing agent,

5   increased the extension rate and stabilized it (Figure 9). This effect is similar to that previously found with cucumber walls (Cosgrove 1989). Inclusion of dithiothreitol throughout the extension period resulted in a simpler decay to a constant extension rate (Figure 10). Pretreatment of the coleoptile wall with boiling water for 15 sec to inactivate wall proteins eliminated the acid-induced

10   extension (Figure 8A), suggesting that extension of these walls may be due to a protein-mediated reaction. These observations are consistent with previous reports of acid-induced extension in oat and cucumber walls.

To pursue this point further, we tested the ability of cucumber expansins to induce extension of oat coleoptile walls. As shown in Figure 11B, they indeed

15   induced extension in oat coleoptile walls, but with less effectiveness than when assayed with cucumber walls. This last point is quantified in Figure 12A, which compares responsiveness of cucumber and oat walls to a partially purified cucumber expansin fraction. The maximal response of cucumber walls was about three times higher than that of oat walls. To obtain the same extension response in

20   oat walls as was elicited in cucumber walls by 2 μg mL^{-1} of cucumber protein, ten times as much protein was required. Figure 12B shows that cucumber walls were similarly more sensitive to the active oat extract than were oat walls. The protein-induced extension was stabilized by inclusion of 1-10 mM dithiothreitol in the incubation buffer (data not shown). We also confirmed that cucumber expansins

25   caused very little extension of barley coleoptiles (data not shown). Thus, we conclude that oat coleoptile walls are substantially more responsive in these reconstitution assays than barley coleoptile walls, but less responsive than cucumber walls.

Despite the quantitative differences, these results show that oat and

30   cucumber proteins can induce qualitatively similar extension responses in oat and

cucumber walls. We thus infer that a similar biochemical mechanism is involved in acid-induced wall expansion in grasses as in dicots.

One might expect that extraction of frozen/thawed coleoptiles with 1 M NaCl might remove most or all of the acid-extension response. Figure 13 shows that overnight extraction of coleoptiles indeed diminished their acid-extension response. This loss could be due to extraction .of the expansins; alternatively, the walls or their proteins may have been modified in some other way so that they lost the acid-extension response. Cucumber walls were previously found to lose their native extension response when they were pre-incubated in neutral pH (Cosgrove 1989). Further work will be needed to differentiate between these and other possible explanations.

To assay the pH dependence of the extractable expansin activity from oats, we used cucumber hypocotyl walls as the "substrate" for measuring the extension activity of oat proteins. Cucumber walls were used because they were easier to prepare, broke less often, had lower baseline extension rates, and proved to be a more sensitive substrate for extension assays than did the walls from oat coleoptiles. The active oat fractions from the DEAE-column had a pH optimum between 4.5 and 5.0 (Figure 14). At pH 3.5 or 5.5, the activity was reduced by about 50%. In contrast, cucumber expansins displayed a broader pH optimum, with high activity maintained at pH 3.5.

When the active proteins from the DEAE-column were passed through a Concanavalin A (Con A) column, the majority of the extension activity did not bind to the lectin column, suggesting that the activity was not associated with glucosyl- or manosyl- glycoproteins. When the active fractions which passed through the Con A column were further fractionated by HPLC on a carboxymethyl cation exchange column, extension activity was eluted as a single major peak at about 15 minutes (Figure 15). The activity of this protein fraction had an acid optimum similar to that shown in Figure 14 (data not shown). SDS-polyacrylamide gel electrophoresis revealed that a protein with molecular mass of about 29 kD was associated with this active fraction (Figure 16). We designate this protein as oat expansin-29 (oat Ex29, oEx-29). Generally, expansins are about 25-30 kD in size.

20

A summary of the purification steps for the oat Ex29 is shown in Table 2. This protein was purified sequentially by ammonium sulfate precipitation, DEAE chromatography, Con A affinity chromatography and CM cation exchange chromatography. If the extension activity in ammonium-sulfate-precipitated proteins is taken as 100%, a purification of S1 fold with 69% yield was obtained after two steps of ion exchange chromatography. Because Con A affinity chromatography did not significantly purify oEx-29, this step was often omitted without significant effect on the purification.

To examine whether expansin activity was also present in the protoplasm of oat coleoptiles, we fractionated the soluble protoplasmic proteins (all proteins in the coleoptile homogenate not bound to the wall) by ammonium sulfate precipitation and DEAE-column chromatography. Little or no activity was detected in any fractions (data not shown), suggesting that the responsible protein was bound to the cell walls of oat coleoptiles.

Because oat Ex29 was capable of catalyzing extension of walls from cucumber, we wished to test whether it is immunologically related to the (apparently) 29-kD cucumber expansin cEx-29. Figure 17 shows that the oat Ex-29 was specifically recognized by an antibody raised against cucumber Ex-29. Pre-immune serum under the same conditions did not label Ex-29 (not shown). Little signal was detected in the soluble protoplasmic fraction of oat coleoptiles, which is consistent with our other evidence that the Ex-29 is a wall-bound protein. When equal amounts of crude and purified wall protein were assayed by Western analysis, the purified protein gave a much greater signal (Figure 17). These results demonstrate that the-extension-inducing activity co-purifies with the protein that is antigenically related to cucumber Ex-29.

Our results show that oat coleoptile walls possess a protein that can mediate acid-induced extension of grass coleoptile walls and dicot walls. This protein resembles the cucumber 29-kD expansin in that it induces extension at acid pH but not at neutral pH, its activity is stabilized by dithiothreitol, it has a similar size as judged by SDS-PAGE, and it is specifically recognized by an antiserum raised against the cucumber protein. Because of these similarities between the oat and

cucumber expansins, we propose that expansins are evolutionarily conserved proteins that underlie at least part of the acid-extension response common to the walls of-many plant species.

The similarity between oat and cucumber expansins surprised us because we found that cucumber expansins caused little extension of maize coleoptile walls and no extension of barley coleoptile walls. We confirmed that barley walls were unresponsive to cucumber expansins, but our positive results with oat coleoptiles show that this insensitivity is not a general property of grass coleoptiles. We do not know why barley walls failed to extend in the presence of cucumber or oat expansins.

The similarity between oat and cucumber expansins is also surprising because the matrix components of the wall are believed to be important for wall loosening and extension, yet these components are quite different for grass walls and dicots. The major matrix polysaccharides of the coleoptile wall are (1->3, 1->4)-β-D-glucans and arabinoxylans whereas dicots contain principally xyloglucans and pectins. Dicot walls contain hydroxyproline-rich glycoproteins of the extensin family whereas grass walls contain much lower amounts of such proteins. It may be that expansins interact directly with wall components common to both types of walls (e.g., cellulose or a minor matrix component) or that they can act on different glycans with similar functions, e.g. xyloglucans and (1->3, I->4)-β-D-glucans.

Although oat Ex29 can induce an acid-dependent extension in coleoptile walls, there are notable differences between the native and reconstituted acid-extension responses in coleoptiles. First, the acid response of native walls includes a large, but transient, burst in extension, which is mostly decayed away by 60-90 minutes. This burst is largely lacking in oEx29-reconstituted extensions, which resemble more closely the steady extensions which outlast the transient (e.g., Figures 8 and 10). Second, the pH dependence of reconstituted extension (Figure 14) does not exactly match the pH dependence reported for acid-extension responses of isolated coleoptile walls. The reconstituted extensions displayed a maximum at pH 4.5 and fell off at lower pH values, whereas acid extensions of native coleoptile walls did not fall off at lower pH values. Third, the maximum

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extension rate inducible with exogenous Ex29 was substantially less than acid-extension responses of native-eoleoptile walls (i.e., about 2 % h^{-1} for reconstituted extensions versus about 5 % h^{-1} for the stable component of native wall extensions or 20-30 % h^{-1} for the immediate response of native-walls).

5   These differences between the reconstituted and native acid-extension responses of coleoptile walls could indicate that oat coleoptiles possess additional acid-extension processes, other than the one mediated by oEx-29. On the other hand, these differences might also result from inadequacies in our reconstitution methods. For example, heat inactivation of the coleoptile wall by treatment with

10   boiling water may modify the wall's structure so that it is not as susceptible to oEx-29 action. There may also be differences due to poor accessibility of exogenous Ex-29 to its site of action in the wall, or with need for ancillary wall enzymes that are inactivated by heat treatment. Hence, we believe that, at this stage, caution is warranted in interpreting the differences between native and reconstituted

15   extensions.

From the characteristics of oEx-29-induced extension of coleoptile walls and the above considerations, we propose that oat Ex29 is responsible for at least part of the long-term (> 1 h) acid-induced extension responses of oat coleoptiles. This view is strengthened by the findings that reconstituted wall extension and

20   endogenous wall extension exhibit similar sensitivities to biochemical activators and inhibitors. In cucumber, exogenous expansins can restore extension rates as high or higher than the long-term extension rates in native walls exposed to acid pH. Although we believe there to be good reasons for thinking that expansins mediate the long-term acid-induced extension of isolated walls, their role in the

25   growth of living tissues has not been directly addressed. This assessment will require experiments in which the action of expansins are specifically inhibited or enhanced. Further studies of the biochemical action of expansins and the genes that encode them may provide the tools for this assessment.

The digestive tract of many animals contains enzymes able to catalyze

30   reactions weakening various chemical bonds. We postulated that the digestive tract of animals which consume green parts of plants may contain proteins of action

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similar to expansins. We found expansin-like proteins in the digestive tract and feces of the snail *Helix aspersa*. Western blot analysis of the snail proteins probed with the antibody raised against cucumber expansins cEx-29 indicates the presence of a protein antigenically similar to cucumber expansin.

5   The expansin-like proteins in the snail digestive tract may be synthesized by the snails or by an endosymbiont living in the digestive system of the snail. Such symbionts might be bacteria, fungi, protists or some other microbe.

We assayed the effects of expansins on the mechanical properties of pure cellulose paper, which derives its strength principally from hydrogen bonding

10   between cellulose microfibrils. Because paper is much simpler in composition and structure than plant cell walls, the action of expansins could be much easier to interpret. S1 protein showed clear effects on the mechanical properties of Whatman number 3 filter paper in both extensometer and stress relaxation assays. As our experiments show expansins are also able to disrupt slick paper (Figure 7).

15   Expansins appeared to weaken cellulose paper in a manner different from that affected by cellulase. While both expansins and cellulase led to breakage of the paper in extensometer assays, only expansins showed a clear enhancement of stress relaxation in this material. Furthermore, incubations of paper with expansins did not lead to the release of reducing sugars, as was seen in incubations with

20   cellulase. Because expansins did not hydrolyze the paper, and because hydrogen bonding accounts for the tensile strength of paper we conclude that expansins disrupt the hydrogen bonding between cellulose fibrils. Expansins probably do not catalyze cell wall loosening by disrupting hydrogen bonds directly between microfibrils, as these structures appear to be separated from one another by matrix

25   polysaccharides which are thought to coat the outside of microfibrils in the wall. However, it is possible that they could induce wall extension by disrupting hydrogen bonding between the matrix polysaccharides and the microfibrils, thus reducing the resistance to movement of the microfibrils.

Stress relaxation assays performed to test the ability of the snail expansin-

30   like protein to weaken the bonds in the paper indicate that this ability is attributed to all expansins and expansin-like proteins.

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In addition to the above, we have cloned, isolated and fully sequenced a cDNA coding for the cucumber cEx-29 (S1 fraction) expansin protein. The sequence is disclosed as the nucleic acid sequence and as an amino acid sequence (see SEQ ID NOS:1 and 7 in the accompanying Sequence Listing), and five other

5   amino acid sequences are also disclosed (two from rice and three from *Arabidopsis*) (SEQ ID N0:2 through SEQ ID N0:6).

The cDNA sequence (SEQ ID NO:1) for the cucumber cEx-29 expansin protein can also include a modification of the cDNA. As used herein, a "modification" of a nucleic acid can also include one or several nucleotide

10   additions, deletions, or substitutions with respect to a reference sequence. A modification of a nucleic acid can include substitutions that do not change the encoded amino acid sequence due to the degeneracy of the genetic code. Such modifications can correspond to variations that are made deliberately, or which occur as mutations during nucleic acid replication.

15   Corresponding cDNA sequences can be determined by methods known in the art, such as by PCR or by screening genomic, cDNA or expression libraries.

Nucleic acid hybridization will be affected by such conditions as salt concentration, temperature, solvents, the base composition of the hybridizing species, length of the complementary regions, and the number of nucleotide base

20   mismatches between the hybridizing nucleic acids, as will be readily appreciated by those skilled in the art. "Stringent hybridization conditions" and "stringent wash conditions" in the context of nucleic acid hybridization experiments depend upon a number of different physical parameters. The most important parameters include temperature of hybridization, base composition of the nucleic acids, salt

25   concentration and length of the nucleic acid. One having ordinary skill in the art knows how to vary these parameters to achieve a particular stringency of hybridization. In general, "stringent hybridization" is performed at about 25° C below the thermal melting point (T_m) for the specific DNA hybrid under a particular set of conditions. "Stringent washing" is performed at temperatures about 5° C

30   lower than the T_m for the specific DNA hybrid under a particular set of conditions. The T_m is the temperature at which 50% of the target sequence hybridizes to a

perfectly matched probe. See Sambrook (1989), supra, hereby incorporated by reference.

The T_m for a particular DNA-DNA hybrid can be estimated by the formula:

$$T_m = 81.5° \text{ C} + 16.6 \ (\log_{10}[\text{Na}^+]) + 0.41 \ (\text{fraction G+C}) - 0.63 \ (\% \text{ formamide}) - (600/1)$$

where 1 is the length of the hybrid in base pairs.

The T_m for a particular RNA-RNA hybrid can be estimated by the formula:

$$T_m = 79.8° \text{ C} + 18.5 \ (\log_{10}[\text{Na}^+]) + 0.58 \ (\text{fraction G+C}) + 11.8 \ (\text{fraction G+C})^2 - 0.35 \ (\% \text{ formamide}) - (820/1).$$

The T_m for a particular RNA-DNA hybrid can be estimated by the formula:

$$T_m = 79.8° \text{ C} + 18.5 (\log_{10}[\text{Na}^+]) + 0.58 \ (\text{fraction G+C}) + 11.8 \ (\text{fraction G+C})^2 - 0.50 \ (\% \text{ formamide}) - (820/1).$$

In general, the T_m decreases by 1-1.5° C for each 1% of mismatch between two nucleic acid sequences. Thus, one having ordinary skill in the art can alter hybridization and/or washing conditions to obtain sequences that have higher or lower degrees of sequence identity to the target nucleic acid. For instance, to obtain hybridizing nucleic acids that contain up to 10% mismatch from the target nucleic acid sequence, 10-1 5° C would be subtracted from the calculated T_m of a perfectly matched hybrid, and then the hybridization and washing temperatures adjusted accordingly. Probe sequences may also hybridize specifically to duplex DNA under certain conditions to form triplex or other higher order DNA complexes. The preparation of such probes and suitable hybridization conditions are well-known in the art.

An example of stringent hybridization conditions for hybridization of complementary nucleic acid sequences having more than 100 complementary residues on a filter in a Southern or Northern blot or for screening a library is 50% formamide/6X SSC at 42° C for at least ten hours and preferably overnight (approximately 16 hours). Another example of stringent hybridization conditions is 6X SSC at 68° C without formamide for at least ten hours and preferably overnight. An example of moderate stringency hybridization conditions is 6X SSC at 55° C without formamide for at least ten hours and preferably overnight. An example of

low stringency hybridization conditions for hybridization of complementary nucleic acid sequences having more than 100 complementary residues on a filter in a Southern or Northern blot or for screening a library is 6X SSC at 42° C for at least ten hours. Hybridization conditions to identify nucleic acid sequences that are similar but not identical can be identified by experimentally changing the hybridization temperature from 68° C to 42° C while keeping the salt concentration constant (6X SSC), or keeping the hybridization temperature and salt concentration constant (e.g., 42° C and 6X SSC) and varying the formamide concentration from 50% to 0%. Hybridization buffers may also include blocking agents to lower background. These agents are well-known in the art. See Sambrook et al. (1989), supra, herein incorporated by reference. See also Ausubel (1992), supra, Ausubel (1999), supra, and Sambrook (2001), supra.

Wash conditions also can be altered to change stringency conditions. An example of stringent wash conditions is a 0.2X SSC wash at 65° C for 15 minutes (see Sambrook (1989), supra, for SSC buffer). Often the high stringency wash is preceded by a low stringency wash to remove excess probe. An exemplary medium stringency wash for duplex DNA of more than 100 base pairs is 1X SSC at 45° C for 15 minutes. An exemplary low stringency wash for such a duplex is 4X SSC at 40° C for 15 minutes. In general, signal-to-noise ratio of 2X's or higher than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization.

As defined herein, nucleic acid molecules that do not hybridize to each other under stringent conditions are still substantially similar to one another if they encode polypeptides that are substantially identical to each other. This occurs, for example, when a nucleic acid molecule is created synthetically or recombinantly using high codon degeneracy as permitted by the redundancy of the genetic code.

Hybridization conditions for nucleic acid molecules that are shorter than 100 nucleotides in length (e.g., for oligonucleotide probes) may be calculated by the formula:

$$T_m = 81.5° \text{ C} + 16.6(\log_{10}[\text{Na}^+]) + 0.41(\text{fraction G+C}) - (600/N),$$

wherein N is chain length and the [Na$^+$] is 1 M or less. See Sambrook (1989), supra. For hybridization of probes shorter than 100 nucleotides, hybridization is usually performed under stringent conditions (5-10° C below the T_m) using high concentrations (0.1-1.0 pmol/ml) of probe. See Sambrook (1989), supra. Determination of hybridization using mismatched probes, pools of degenerate probes or "guessmers," as well as hybridization solutions and methods for empirically determining hybridization conditions are well-known in the art. See, e.g., Ausubel (1999), supra; Sambrook (1989), supra.

DNA sequences can be used in expression systems to obtain large quantities of expansin(s) for commercial applications. Moreover, the DNA sequences may be modified to obtain expansins with improved properties such as different pH dependence, stability, substrate specificity, activity, and so on. The DNA sequences may be used to identify and to isolate other naturally-occurring expansin genes with improved or novel properties, and the corresponding proteins expressed by such genes.

An expansin, according to the present invention, is a purified protein generally having greater than about 60% sequence similarity and preferably greater than about 70% sequence similarity with the amino acid sequence disclosed in SEQ ID N0:7. In a corresponding manner, the applicable DNA sequence that expresses an expansin will have about 60% sequence similarity, and preferably greater than about 70% sequence similarity, with the nucleic acid sequence disclosed in SEQ ID NO:1. The amino acid sequences identified in SEQ ID NO:2 through SEQ ID NO:6 are examples of sequences which meet the sequence similarity definition and thus are examples of expansins.

A "modification" of an expansin polypeptide also encompasses conservative substitutions of an expansin polypeptide amino acid sequence. Conservative substitutions of encoded amino acids include, for example, amino acids that belong within the following groups: (1) non-polar amino acids (Gly, Ala, Val, Leu, and Ile); (2) polar neutral amino acids (Cys, Met, Ser, Thr, Asn, and Gln); (3) polar

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acidic amino acids (Asp and Glu); (4) polar basic amino acids (Lys, Arg and His); and (5) aromatic amino acids (Phe, Trp, Tyr, and His).

Those of ordinary skill in the art will recognize that some substitution will not alter the expansin activity of the polypeptide to an extent that the character or nature of the expansin polypeptide is substantially altered. A "conservative substitution" is one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydropathic nature of the polypeptide to be substantially unchanged. Modifications may be made in the structure of the polynucleotides and polypeptides of the present invention and still obtain a functional molecule that encodes a variant or derivative polypeptide with desirable characteristics, e.g., with expansin-like characteristics. When it is desired to alter the amino acid sequence of a polypeptide to create an equivalent, or an expansin variant or portion of a polypeptide of the invention, one skilled in the art will typically change one or more of the codons of the encoding DNA sequence according to Table 1 (See infra). For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of expansin activity. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence, and, of course, its underlying DNA coding sequence, and nevertheless obtain a protein with like properties. It is thus contemplated that various changes may be made in the peptide sequences of the disclosed compositions, or corresponding DNA sequences which encode said peptides without appreciable loss of their biological utility or activity. A degenerate codon means that a different three letter codon is used to specify the same amino acid. For example, it is well known in the art that the following RNA codons (and therefore, the corresponding DNA codons, with a T substituted for a U) can be used interchangeably to code for each specific amino acid:

TABLE 1

Amino Acids	Codons
Phenylalanine (Phe or F)	UUU, UUC, UUA or UUG
Leucine (Leu or L)	CUU, CUC, CUA or CUG
Isoleucine (Ile or I)	AUU, AUC or AUA
Methionine (Met or M)	AUG
Valine (Val or V)	GUU, GUC, GUA, GUG
Serine (Ser or S)	AGU or AGC
Proline (Pro or P)	CCU, CCC, CCA, CCG
Threonine (Thr or T)	ACU, ACC, ACA, ACG
Alanine (Ala or A)	GCU, GCG, GCA, GCC
Tryptophan (Trp)	UGG
Tyrosine (Tyr or Y)	UAU or UAC
Histidine (His or H)	CAU or CAC
Glutamine (Gln or Q)	CAA or CAG
Asparagine (Asn or N)	AAU or AAC
Lysine (Lys or K)	AAA or AAG
Aspartic Acid (Asp or D)	GAU or GAC
Glutamic Acid (Glu or E)	GAA or GAG
Cysteine (Cys or C)	UGU or UGC
Arginine (Arg or R)	AGA or AGG
Glycine (Gly or G)	GGU or GGC or GGA or GGG
Termination codon	UAA, UAG or UGA

Expansins equivalent to those described by this definition can be identified by their expansin characteristics, as explained throughout this specification, and all expansins are thus believed to be patentable equivalents.

PCR Technology

Polymerase Chain Reaction (PCR) is a repetitive, enzymatic, primed synthesis of a nucleic acid sequence. This procedure is well known and commonly used by those skilled in this art (see U.S. Pat. Nos. 4,683,195, 4,683,202, and 4,800,159; Saiki et al., 1985). PCR is based on the enzymatic amplification of a DNA fragment of interest that is flanked by two oligonucleotide primers that hybridize to opposite strands of the target sequence. The primers are oriented with the 3' ends pointing towards each other. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary sequences, and extension of the annealed primers with a DNA polymerase result in the amplification of the segment defined by the 5' ends of the PCR primers. Since the extension product of each primer can serve as a template for the other primer, each cycle essentially

doubles the amount of DNA fragment produced in the previous cycle. This results in the exponential accumulation of the specific target fragment, up to several million-fold in a few hours. By using a thermostable DNA polymerase such as Taq polymerase, which is isolated from the thermophilic bacterium Thermus aquaticus,

5   the amplification process can be completely automated. Other enzymes that can be used are known to those skilled in the art.

The polynucleotide sequences of the subject invention (and portions thereof such as conserved regions and portions that serve to distinguish these sequences from previously-known sequences) can be used as, and/or used in the design of,

10   primers for PCR amplification. In performing PCR amplification, a certain degree of mismatch can be tolerated between primer and template. Therefore, mutations, deletions, and insertions (especially additions of nucleotides to the 5' end) of the exemplified polynucleotides can be used in this manner. Mutations, insertions and deletions can be produced in a given primer by methods known to an ordinarily

15   skilled artisan.

All references and publications cited herein are expressly incorporated by reference into this disclosure. There are a variety of alternative techniques and procedures available to those of skill in the art which would similarly permit one to successfully perform the intended invention in view of the present disclosure. It

20   will be appreciated by those skilled in the art that while the invention has been described above in connection with particular embodiments and examples, the invention is not necessarily so limited and that numerous other embodiments, examples, uses, modifications and departures from the embodiments, examples and uses may be made without departing from the inventive scope of this application.

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EXAMPLE 1
Preparation of Plant Materials

Seeds of cucumber (Cucumis sativus cv Burpee Pickler) were sown on Seed

30   Germination Kimpack Paper K-22 (Seedburro Equipment Corporation, Chicago, Illinois) soaked with distilled water, in flats, 50 x 25 x 6 cm, with lids of the same dimensions. Seedlings were grown in the dark for 4 days at 27°C. The apical 3 cm

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of hypocotyl was excised and frozen at -20°C for no more than 5 days and prepared for creep measurements as previously described (Cosgrove 1989). For bulk wall extractions, the apical 3-cm hypocotyl regions were collected on ice water and homogenized with 25 mM sodium acetate, 2 mM EDTA, pH 4.5 in a Waring blender. The wall fragments were collected and washed twice by filtration through Miracloth and subsequently used for protein extraction. Basal walls were from the lower 6 cm of the (15 cm long) hypocotyls.

Oat seedlings were grown in moist vermiculite in complete darkness at 27°C. Except as noted, coleoptiles were from 4-day old oat seedlings (*Avena sativus L.* cv. Olge, from Carolina Biological Supply, Burlington, NC, USA).

Seedlings were quickly harvested under room lights. For wall extension assays, the apical 2 cm region of the growing stem or coleoptile was excised, sealed in aluminum foil and frozen at -20°C prior to use. Coleoptiles were separated from primary leaves. Cuticles were abraded by rubbing the coleoptiles or stems between two fingers coated with a slurry of carborundum (320 grit, well washed prior to use; Fisher Scientific, Fair Lawn, NJ). For oat coleoptiles, the cuticle was generally abraded prior to freezing, whereas for cucumber and pea stems the frozen segment was quickly abraded. In some instances as noted, coleoptile cuticles were removed by stripping the epidermis from the tissue with fine forceps and the remaining coleoptile cylinder was bisected longitudinally prior to freezing. Tissues were thawed, pressed under weight for 5-10 minutes to remove tissue fluids and clamped in an extensometer (5 mm between the clamps, corresponding to the apical 3-8 mm of the stem or coleoptile).

Seeds of pea (*Pisum sativum* cv Alaska), radish (*Raphanus sativus* cv Crimson Giant), tomato (*Lycopersicon esculentum* cv Rutgers), onion (*Alium cepa* cv Snow White), maize (*Zea mays* cv B73xMol7), and barley (*Hordeum sativum* cv Barsoy) were sown on vermiculite wetted with distilled water and grown for 4 days in the dark at 27°C. Hypocotyls of pea, radish and tomato; primary leaves of onion; and coleoptiles of maize and barley were excised and frozen for subsequent extension experiments. Young growing leaves of zephyr lily (*Zephyranthes Candida*) were removed from a greenhouse-grown colony of plants which were

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kept in the dark at 27°C for 12 hr prior to harvest, and frozen for later extension assay.

EXAMPLE 2
Isolation of Expansins cEx-29 and cEx-30 from Cucumber Protein Extraction

Washed cucumber cell wall fragments (from 150-200 g of tissue) were extracted overnight in 20 mM Hepes, pH 6.8, 1 M NaCl at 4°C. Cell wall fragments were-filtered on Miracloth and the salt-solubilized fraction precipitated with ammonium sulfate (the activity precipitated between 20 and 60% saturation with $[NH_4]_2SO_4$). The precipitate was desalted on a 7-mL column of Bio-Gel P2 (BioRad Laboratories) into 50 mM sodium acetate, pH 4.5. Protein concentration was 2 to 4 mg/mL, estimated by Coomassie Protein Assay Reagent (Pierce, Rockford, Illinois).

For the comparison of soluble and wall associated proteins, 100 g of tissue was harvested and homogenized with 100 mL of 25 mM sodium acetate, pH 4.5, 1 mM EDTA. Wall fragments were filtered out and the remaining solution designated as the soluble fraction. Wall fragments were cleaned as described above, and then extracted in 200 mL of 20 mM Hepes, pH 6.8, 1 M NaCl for one hour at 40°C. Aliquots of both solutions were dialyzed against 50 mM sodium acetate pH 4.5 and tested for reconstitution activity, as described below.

Protein Fractionation

The 20 to 60% $(NH_4)_2SO_4$ precipitate pellet was resuspended in 2 mL of distilled water and insoluble material was removed by centrifugation and filtration through a Centricon 30 microconcentrator (Amicon, Beverly, Massachusetts) prior to being loaded onto a C3 hydrophobic interactions column (ISCO C-3/6.5 μm 10x250 mm) equilibrated with 50 mM sodium acetate, pH 4.5, 20% (saturation) $(NH_4)_2SO_4$. Proteins were eluted from the column with a linear gradient from the equilibration buffer into 50 mM sodium acetate, pH 4.5, in 35 minutes. at a flow rate of 1 mL/minutes. Fractions were desalted on a 7-mL column of Bio-Gel P-2 (Bio-Rad Laboratories, Richmond, California) into 50 mM sodium acetate and

assayed for their ability to reconstitute extension to inactivated cucumber hypocotyls sections.

The active fractions from the C3 column were pooled and subsequently desalted and concentrated on a Centricon 30 microconcentrator (Amicon, Beverly, Massachusetts), the buffer being exchanged for 15 mM Mes NaOH, pH 6.5. The concentrated sample was then loaded (in a volume of 1.7 mL) onto a sulfopropyl cation exchange column (Bio-Rad HRLC MA7S 50x7.8 mm) equilibrated with 15 mM Mes NaOH, pH 6.5, and proteins eluted with an ascending gradient of NaCl (from 0 to 1.0 M over 45 minutes) in the same buffer at a flow rate of 1 mL/min. A_{280} nm of eluting proteins was measured using a Dionex Variable Wavelength Detector (VDM-2) .

Active fractions from ammonium sulfate precipitation, C3 column and sulfopropyl fractions S1 and S2 were concentrated, desalted on Centricon 30 Microconcentrators, and then run on SDS-polyacrylamide gels, according to the method of Laemmli. Gels were subsequently stained with Coomassie Brilliant Blue R250. Fractionation by this procedure was repeated more than 10 times with similar results. Results are presented in Figure 2.

EXAMPLE 3
Isolation of Expansins oEx-29 from Oat Protein Extraction

For oat protein extraction, oat seedlings were rapidly cut under room lights and placed in ice water. The apical 2.5 cm (+1- 0.5 cm) of each coleoptile was then cut, separated from the primary leaf, and placed on ice while the other coleoptiles were harvested. About 500 coleoptiles were homogenized in 200 mL of 10 mM sodium phosphate, pH 6.0. In some instances the coleoptiles were collected in lots of 100-200 and frozen (-20°C) for 1 to 3 days prior to homogenization. The homogenate was filtered through a nylon screen (70 mm mesh), and the cell walls were collected and washed 4 times by resuspending in the homogenization buffer (300 mL) followed by filtration. Ionically-bound proteins were extracted for at least 1 hour at 4°C with 50 mL of 1 M NaCl containing 20 mM Hepes (pH 6.8), 2 mM EDTA and 3 mM sodium metabisulfite. Wall fragments were removed by filtration or centrifugation and the wall proteins in the supernatant were precipitated

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with ammonium sulfate (0.4 g added to each mL). Precipitated proteins were dissolved in 1.5 mL of water and desalted on an Econo-Pac 10DG desalting column (Bio-Rad Laboratories, Richmond, CA, USA) which was equilibrated with 20 mM Tris-HCl, pH 8.0, containing 100 mM NaCl. Without NaCl the active proteins tended to bind to the desalting column, resulting in a lower recovery.

Protein Fractionation

Protein solution from the desalting column was centrifuged in a microcentrifuge for 3 minutes to remove precipitates. Proteins were then loaded onto a DEAE-column (Sephadex A-25, Sigma) equilibrated with 20 mM Tris-HCl, pH 8.0, containing 100 mM NaCl at 25°C. The proteins bound to the DEAE-column were eluted by 1 M NaCl in 20 mM Tris-HCl buffer, pH 8.0.

A 1-mL Concanavalin A column (Sigma) was equilibrated with 200 mM NaCl containing 1 mM each of Mg^{+2}, Ca^{+2}, and Mn^{+2}. Proteins from the DEAE-column were passed through this column. After the column was washed extensively with the same solution, the bound proteins were eluted with the same solution containing 6.5 M a-methyl manoside. Extension activity was associated with the fractions that did not bind to the column.

Active fractions were further separated by HPLC using a carboxymethyl (CM) cation exchange column (4.6 x 250 mm, CM300/6.5 mm, ISCO, Nebraska) equilibrated with 10 mM Mes, pH 5.5. Before the protein sample (1 mL) was loaded on the column, the sample buffer was exchanged for 10 mM MES, pH 5.5, by use of a 30-kD Centricon microconcentrator (Amicon, Beverly, MA, USA). Proteins were eluted from the CM column at a flow rate of 1 mL/min with a gradient of 0 to 0%, 0 to 4%, 4 to 6% and 6 to 100% of 1.0 M NaCl in 10 mM MES, pH 5.5, from 0 to 5 minutes, 5 to 10 minutes, 10 to 30 minutes and 30 to 50 minutes, respectively, and detected by absorbance at 280 nm.

Proteins were quantified colorimetrically using the Coomasie Protein Assay Reagent (Pierce, Rockford, IL, USA). For SDS-PAGE (Laemmli 1970), proteins were separated on a 14% polyacrylamide gel or a 4-20% gradient polyacrylamide gel (Bio-Rad Ready Gel, Richmond, CA, USA). For Western analysis, proteins

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were electrophoretically transferred to a nitrocellulose membrane in a solution of 192 mM glycine, 25 mM Tris, 20% methanol (v/v) at 10 volt/cm for 3 hours or in some cases 16 hours. After the membrane was blocked with 3% bovine serum albumin in phosphate buffered saline containing 0.05% Tween-20 (PBST), it was incubated for 2 hours in PBST containing antiserum (1:3000 dilution). The membrane was washed 4 times with FBST and then incubated for 1 hour with goat anti-rabbit IgG-conjugated alkaline phosphatase (Sigma; dilution of 1:4000) in PBST. The Western blot was developed using bromochloroindolyl phosphate/nitro blue tetrazolium and the reaction was stopped with 10 mM EDTA.

Table 2.

Purification of oat Ex29 from etiolated oat coleoptiles

Activity was assayed as described in Figure 4 and expressed as the initial increase in the extension rate of isolated cucumber cell walls upon addition of the protein fraction (e.g., 5 to 30 minutes after protein addition). Total activity was calculated by dividing the activity (measured in 1-3 extension assays) by the fraction of protein used for each assay. Specific activity was calculated by dividing the total activity by the total protein.

Purification Step	Total Protein	Total Activity units (μm/min)	Specific Activity (units/mg	Purification (fold)	Yield (%)
$(NH_4)_2SO_4$ precipitation	444	123	278	1	100
DEAE	190	104	546	2	84
CM-HPLC	6	85	14278	51	69

Soluble protoplasmic proteins were obtained by homogenizing oat coleoptiles in 10 mM sodium phosphate buffer (in some cases, 10 mM Hepes, pH 6.8) and centrifuging at 4°C at 26,000 g for 10 minutes to remove particulate matter.

EXAMPLE 4
Preparation of Expansin-like Proteins from Snails and Snail Feces

Acetone powders of the visceral humps of Helix pomatia were purchased from Sigma Chemical Company. Live snails (*Helix aspersa*) were purchased from NASCO. Protein solution was made by dissolving snail acetone powder (10 mg/ml) in 50 mM sodium acetate buffer, pH 4.5. Feces of Helix aspersa were dissolved in the same buffer.

EXAMPLE 5
Extension Measurements

Extension measurements were performed using a constant load extensometer as described by Cosgrove (1989). Briefly, frozen/thawed tissues were abraded for a short time with carborundum to disrupt the cuticle, boiled in water for 15 seconds (for reconstitution assays), and secured between two clamps (with about 5 mm between the clamps) under a constant tension of 20 g force (for assays with radish, tomato and onion tissues the tension was reduced to 10 g). Plastic cuvettes were fitted around the walls and filled with bathing solution. For reconstitution assays the bathing solution was first 50 mM sodium-acetate pH 4.5 for 30 minutes, followed by the protein fraction to be assayed in the same, or equivalent pH buffer. Movement of the lower clamp was detected with a position transducer and recorded on a microcomputer. All extension assays reported here were repeated at least five times, except for the heterologous reconstitutions *(e.g.* with walls from pea, tomato and corn), which were performed three to five times.

Cucumber Expansins Activity

Extension induced by cucumber expansins is presented in Figures 1, 11, 12.

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Oat Expansins Activity

Extension induced by oat expansin is presented in Figures 11 and 12.

5   Snails Expansins Activity

Extension induced by snail expansin-like proteins is presented in Figures 18 and 19. The experiment was performed as described for cucumber and oat proteins.

The protein solution obtained from snail acetone powder by dissolving snail

10   acetone powder (10 mg/mL) into 50 mM sodium acetate, pH 4.5, is capable of inducing extension of isolated cucumber walls.

EXAMPLE 6
Effects of DTT and Metal Ions on Endogenous Acid Induced Wall Extension

15   Endogenous acid-induced extension was previously shown to be sensitive to a number of exogenously applied factors (Cosgrove D.J., *Planta* vol. 177, pp 121-130, 1989). The thiol reducing compound dithiothreitol (DTT) was shown to stabilize or even enhance the extension of isolated cucumber cell walls at acid pH. To see if this was a characteristic of our extension inducing proteins, we first

20   reconstituted wall specimens with an active C3 fraction from cucumber, as shown in Figure 4A, stems were allowed to extend for one hour at which point sufficient DTT was applied to bring the bathing solution to 10 mM. Almost immediately the rate of extension is increased and thereafter remained constant, whilst reconstituted sections without the addition of DTT typically show a gradual decline in the rate of

25   extension.

In contrast heavy metal ions including copper and aluminum were shown to be strongly inhibitory to endogenous acid-induced extension (Cosgrove 1989). Again, in Figure 4A, boiled stem tissue reconstituted with C3 proteins showed very clearly the same sensitivity to an application of these ions, the rates of extension

30   falling rapidly to the rate seen in the inactivated tissue before reconstitution.

EXAMPLE 7
Effects of Boiling in Methanol or Hater on the Activity of cucumber Expansins

An unusual property of the endogenous cell wall extension activity is its
ability to survive boiling in methanol (Cosgrove, 1989). We found that methanol-
boiled walls retained extractable activity, whereas extracts from cucumber tissues
boiled in water do not exhibit this activity, shown in Figure 4A. Fractionation of
the active extract from methanol boiled walls by the methods given above showed it
to contain the 29- and 30-kD proteins in the S1 and S2 fractions (data not shown).
The ability of the activity to survive boiling methanol may be due to the small size
of these proteins and to protection within their carbohydrate-rich sites within the
cell wall.

Protein concentrations were estimated using Coomassie Protein Assay
Reagent (Pierce, Rockford Illinois) using a standard curve constructed using BSA
(Pierce). Wall specimens inactivated by boiling were preincubated with S1 and S2
proteins at estimated concentrations of 5 micrograms per mL and then extended in
the extensiometer, as described above, in 50 mM sodium acetate pH 4.5. After 1
hour enough dithiothreitol (DTT), from a 100 mM stock, was added to bring the
bathing solution to 10 mM DTT.

To study the effect of heavy metal ions wall specimens were extended
initially in 50 mM sodium acetate, pH 4.5. After 20 minutes the bathing solution
was exchanged for one containing an estimated 50 micrograms of active C3
proteins. One hour after the start of the experiment $AlCl_3.6H_2O$ or $CuCl_2.2H_2O$,
from 100 mM stocks, were supplied to give a final concentration of 1 mM.

For both treatments 100g (fresh weight) of apical tissue was harvested and
either boiled for 30 seconds in 1 L of distilled water, or for three minutes in 1 L of
Methanol. Methanol boiled tissue was allowed to dry before being resuspended in
50 mM sodium acetate, pH 4.5. Both sets of tissue were then homogenized,
cleaned and salt extracted, as described above. Salt extracts were precipitated with
$(NH_4)_2SO_4$, resuspended and desalted, and tested for reconstitution activity as
described above.

EXAMPLE 8
Effect of Protease Treatments on the Activity of Cucumber Expansins

5   Cosgrove (1989) also showed that endogenous wall extension was eliminated by incubation with proteases, one of the initial clues that extension was catalyzed by proteins. The proteinaceous nature of our extension inducing factors was indicated by the sensitivity of reconstituted extension to protease incubations. This data is presented in Figure 4B; 4-hour incubations with chymotrypsin, papain, pronase and trypsin all reduced extension to rates seen in inactivated tissues.

10   Inactivated stem sections were incubated with active C3 proteins, at an estimated concentration of 50 micrograms per mL, for 30 minutes at 25°C. Reconstituted stems were then incubated with 1000 units of trypsin or 1000 units of chymotrypsin in 50 mM Hepes-NaOH, pH 7.3 for 4 hour at 30°C, and with pronase (2 mg/mL), or papain (2 mg/mL) in 50 mM sodium acetate, pH 5.0, 1 mM DTT for
15   4 hours at 30°C. For a control C3 proteins were incubated at 30°C for 4 hours without the addition of proteases. After incubation the stem sections were assayed for extension, initially in 50 mM Hepes, pH 6.8, after 30 minutes the buffer was switched to 50 mM sodium acetate, pH 4.5. The difference in the rates of extension (pH 4.5 - pH 5.5) was calculated. All experiments were repeated four
20   times.

EXAMPLE 9
Effects of pH on Reconstituted Extension Induced by Cucumber Expansins

25   Reconstitution experiments were carried out at a range of values of pH, results presented in Figure 5 show that there is little or no induction of wall extension by the C3 proteins until between pH 5.5 and pH 5.0 and that extension activity has a maximum somewhere between pH 4.5 and pH 3.5. The region of highest response to changes in pH lies in the range pH 5.5 and pH 4.5. These
30   values are within the range of apoplastic pH values reported in elongating tissues, and support the possibility that these proteins could play an important role in the acid-growth response.

Buffers in the range pH 3.0 to pH 6.5 were prepared by titrating 50 mM citric acid with 1 M K_2HPO_4. Inactivated stem sections were suspended in the extensiometer in buffer of appropriate pH. After 30 minutes of extension the bathing solution was replaced by a 1:1 dilution of C3 proteins (estimated final concentration 50 micrograms per mL) with the appropriate buffer, where necessary the pH was adjusted using either I M citric acid or 1 M K_2HPO_4. Change in rate of extension was calculated as the difference in extension rates before and after addition of proteins. All experiments were repeated eight times.

EXAMPLE 10
        Effects of Cucumber Expansins on Cellulose Filter

Paper strips of Whatman number 3 filter paper (Whatman Lab Sales, Hillsboro, OR) (2 mm by 10 mm) were cut and were clamped in the constant load extensometer as described for cucumber hypocotyl sections. Extension was measured in 50 mM sodium acetate and in the same buffer containing protein fractions. Additionally, effect of expansins on the lost of mechanical strength of paper was measured by stress relaxation assay. For stress relaxation measurements, the paper was incubated in various pretreatment solutions and assayed while still wet, in the standard method.

In extensometer assays S1 protein induced a brief period of extension prior to rapid breakage of the paper, an effect not seen in incubations with buffer alone or with boiled protein. Incubations of paper strips with cellulase showed a similar effect. However, it required at least 100 mg of cellulase to weaken the paper sufficiently to be detected in this assay, whereas only 5 mg of S1 protein produced similar effects. In parallel experiments, cellulase (100 mg/mL) was shown to release 25 mmol reducing equivalents per mL during a five hour incubation with Whatman No. 3 paper. In contrast, no sugar release was detectable in similar assays using S1 and S2 protein (both 5 mg/mL). The results are presented in Figure 6.

S1 protein (5 mg/mL) caused a marked increase in the relaxation rate of paper strips, while cellulase (100 mg/mL) had almost no effect. In both

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extensometer assays and stress relaxation tests, S1 protein which had been boiled for two minutes did not induce the effects apparent with native S1 proteins, indicating that the effects were associated with the activity of the protein. Similar results were obtained in assays with S2 proteins (data not shown).

5   These data indicate that expansins substantially weaken cellulose paper in a manner which does not involve the hydrolysis of cellulose. Since the structural integrity of cellulose paper appears to result from hydrogen bonding between cellulose fibrils, it seems likely that these proteins weaken paper by disrupting these associations.

10   EXAMPLE 11
Effect of Cucumber Expansins on Slick Paper

          In order to test whether a protein preparation containing cucumber expansins can disrupt slick paper, in a similar fashion as it acts on pure cellulose
15   paper the following experiments were conducted. Slick paper was obtained from three sources: (a) the pages of "Nature" magazine (unprinted margins were used); (b) a commercial catalog for chromatography equipment; and (c) a colored advertising insert (Hills) in the Sunday paper. Paper strips of 2-3 mm width were cut and hung in our extensometer, such that a S-mm long strip was put under 20-g
20   force. The buffer contained 50 mM sodium acetate, pH 4.5 with or without added C3 protein (about 20 µg per paper strip was used). The length of the paper strips was recorded for up to 5 hour. Four samples of each paper were used with each treatment (+1- protein). The untreated (control) paper showed very little change in length, and maintained its mechanical strength throughout the test period. In
25   contrast, the treated papers began to extend and quickly broke once extension began. The time for breakage after addition of protein varied from 45 minutes to 2.5 hours, but all samples broke. Figure 7 illustrates the typical extension.

EXAMPLE 12
30   Effect of Snail Expansin-like Proteins on Cellulose Paper

          A stress relaxation assay was performed as described in Example 10. Increased relaxation in time domaines of 10-100 msec (faster relaxation in

comparison with controls) proved the ability of snail expansin-like proteins to weaken the mechanical strength of paper.

EXAMPLE 13

5   Preparation of Antibodies to Cucumber Expansin cEx-29

Antiserum with specific recognition of this protein was raised in a female New Zealand White rabbit by subcutaneous injections of cucumber Ex29 with Freund's adjuvants (Harlow and Lane 1988). Serum dilutions in the range of

10   2000:1 to 4000:1 proved optimal for Western analyses of cucumber and oat proteins.

EXAMPLE 14

Western Blot Analysis of Immunoreactivity between Cucumber and Oat Expansins

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We performed Western analysis of oat coleoptile proteins probed with antiserum against cucumber Ex29. Results are presented in Figure 17. Comparison of lane 1 (crude wall protein) with lane 2 (crude protoplasmic protein) shows that the antiserum recognizes an Ex29-like protein (arrow) specifically bound to the

20   coleoptile cell wall. Comparison of lane 3 (crude wall protein) with lane 4 (purified protein with extension activity) shows that the Ex29-like protein co-purifies with the extension activity. Lane 1 was loaded with 15 μg of crude wall protein (ammonium sulfate precipitate of 1 M NaCl extract). Lane 2 had 15 μg of the soluble protoplasmic protein fraction. These samples were separated on the same-

25   4-20% gradient SDS polyacrylamide gel, blotted onto nitrocellulose, and probed with rabbit antiserum against cucumber Ex29. Lane 3 was loaded with 0.2 μg of crude wall protein and Lane 4 had 0.2 μg of active oat wall-extension protein purified sequentially by DEAE Sephadex and CM-HPLC. These samples (3-4) were separated on the same 14% SDS polyacrylamide gel, blotted onto

30   nitrocellulose, and probed with antiserum. This assay, with minor variations, was carried out four times with similar results.

EXAMPLE 15
Western Blot Analysis of Immunoreactivity between Expansin-like Protein from Snail and Cucumber Expansin cEx-29

5   Fig. 20 presents a Western blot of active HPLC-separated protein fractions from snail acetone powder, probed with antibody PA1 which was raised against cucumber expansin-29. The active fractions show a striking band at about 26 kD, which is similar to (though slightly smaller than) cucumber expansin-29. This result provides strong evidence that the wall extension activity found in the snail

10   acetone powder is due to a protein with similar antigenic determinants as cucumber expansin-29.

      Fig. 21 presents a Western blot of the proteins from Helix aspersa feces, probed with the antibody PA1. The single band indicates the presence of a protein antigenically similar to cucumber expansin-29.

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EXAMPLE 16
Effects of Urea on Plant Cell Wall Extension

      This example shows the effects of 2 M urea on native cell walls, walls

20   reconstituted with expansin, and heat inactivated walls. Native cell walls or heat-inactivated cell walls from cucumber hypocotyls were obtained as earlier described and clamped in the extensometer in 50 mM sodium acetate at pH 4.5. At about 20 minutes, an S1 fraction of cucumber expansin (10 µg/mL) was added to one set of heat-inactivated walls. At about 45 minutes the incubation solutions were replaced

25   with 2 M urea buffered with 50 mM sodium acetate, pH 4.5. The aforesaid solutions also contained 2 mM dithiothreitol to stabilize the expansin activity. Results of this experiment are presented in Figure 22. Data are representative of four (4) trials for each respective treatment. The dotted line of Figure 22 represents native cucumber cell walls.

30   These data show that 2 mM urea acts synergistically with expansins to enhance the extension rate of native cucumber cell walls and heat-inactivated cell walls reconstituted with purified expansins. As indicated in the trace labeled "heat-inactivated" in Figure 22, urea alone (without active expansins in the cell walls) has little effect.

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These results support the contention that expansins disrupt hydrogen bonding between cell wall polymers.

EXAMPLE 17
5   Identification of Expansins in Various Plant Materials

Using the methods earlier described herein for extraction of expansins from cucumber and oat materials, quantities of the following plant materials were treated:

10   (i)   broccoli flower stalks (*Brassica oleracea italica*);

           (ii)   celery petioles (*Apium graveolens*);

           (iii)   tomato leaves (*Lycopersicum esculentum*);

           (iv)   cotton fibers (*Gossypium*); and

           (v)   corn coleoptiles (*Zea mays*).

15   Expansins were identified and confirmed by use of the antibody assay described herein (having common antigenic epitomes with the antibody against Ex29), and the extension activity analysis described herein.

EXAMPLE 18
20   RNA Extraction, PCR Amplification, and cDNA Library

RNA was extracted from 3-cm apical hypocotyls sections from 4-day etiolated cucumber seedlings. The segments were frozen in liquid nitrogen and extracted according to published methods (Chomcynski et al., *Anal. Biochem.* 162,
25   156-159 (1987)). Poly(A)+ RNA was selected by standard procedures (Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed. (1989)) and used for cDNA synthesis with reverse transcriptase (RT) (Gibco/BRL; type M-MLV). A cDNA library was constructed with the λ ZAP II vector, using the Stratagene ZAP cDNA synthesis kit.
30   Degenerate primers based on the N-terminal amino acid sequence from the cucumber S1 expansin were used to amplify a 95-bp cDNA by PCR (sense primer, 5'-GAYTAYGGNGGNTGGCA-3' (SEQ ID NO:8); antisense primer, 5'-TANARRTTNCCRTANCC-3') (SEQ ID NO:9). This product was used as

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template for nested amplification with the primer 5'-CAYGCNACNTTYTAYGG-3' (SEQ ID NO:10) and the same antisense primer, resulting in a 71-bp product, which was cloned into the Invitrogen TA cloning vector. The 71-bp product was radiolabeled by PCR with [α-^{32}P] dCTP, purified by centrifugation through a Bio-Gel P-6 column, and used to screen the cucumber cDNA library. Immobilization of bacteriophage and hybridization were_carried out as described and are well known to those skilled in the art. References disclosing such methods include as described. Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed. (1989). Bluescript SK phagemids containing positive inserts were rescued from λ ZAP II using Exassist helper phage (Stratagene).

For S2 expansin, we designed S2-specific degenerate primers; the sense primer 5'-TTYACNGCNWSNGGNTGGGC-3' (SEQ ID NO:11) was based on the S2 N-terminal peptide; the antisense primer 5'-CCNCCNCKRTADATNC-3' (SEQ ID NO:12) was based on two peptide sequences (S2-22 and S2-55), which we predicted to be contiguous on the basis of homology with the S1 sequence (see Results and Discussion). Amplification with these primers was followed with a nested PCR amplification with the same antisense primer and a different sense primer (5'-CCNCCNCKRTADATNC-3') (SEQ ID NO:13), which was based on the most conserved nucleotide sequence in the alignments of cucumber S1 expansin with several *Arabidopsis* and rice expansin cDNAs. The amplification yielded a product of the predicted size (~300 bp), which was cloned and sequenced to confirm an S2-specific partial clone of 312 bp. This fragment was used to screen the cDNA library.

DNA Sequencing

Plasmid DNA containing cDNA inserts was prepared by the Terrific Borth method (Lee et al., *BioTechniques* 9, 676-679 (1990) and used for dideoxynucleotide sequencing with Sequenase (United States Biochemical). We used subcloning and primer walking strategies to obtain complete sequences.

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SUMMARY AND CONCLUSIONS

In summary, cucumber expansins appear to associate with the cellulose fraction of the cell wall. They do not exhibit polysaccharide hydrolysis under a number of assay conditions and they do not cause a progressive weakening of the wall. Expansins also appear to disrupt hydrogen bonds as particularly noted with cellulose paper.

Isolation of new proteins with new activities often opens new possibilities of application of same. Although the potential applications in paper industry have been emphasized in this disclosure, numerous other directions of use can be imagined. For example expansins could be used for processing of polysaccharides for control of physical properties. Hydrogen bonding is an important determinant of many physical properties of commercial products containing polysaccharides. Expansins may be incorporated into the polysaccharide products to modify hydrogen bonding and thereby modify the physical characteristics of the products. Examples include control of the viscosity and texture of polysaccharide thickeners used in foods and chemical products; control of stiffness and texture of paper products; and control of mechanical strength (e.g. tear strength) of paper products.

The present invention is believed broadly applicable to alteration of various physical properties of polysaccharides. While plant polysaccharides represent a preferred embodiment of the invention, expansins of the invention are believed useful catalytic proteins for the treatment polysaccharides from a variety of sources (i.e., synthetic, bacterial or other microbial system, etc.). While it is reasonable to make a strong claim that expansins primarily operate by disruption of hydrogen bonds, the invention is not necessarily limited to this mode of action. Results concerning altered physical properties of polysaccharides are nonetheless produced by use of the novel expansins of the invention.

Expansins may be used for de-inking paper, which is a significant limitation in current paper recycling operations. In this application, expansins may help loosen the bonding between surface polymers, which are stuck to the ink, and the remainder of the cellulose fibers. Also, in the paper industry expansins may prove useful for large scale paper dissolution, or perhaps for alteration of the mechanical

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properties of dry paper. Treatment of dry paper could produce paper with novel properties.

Expansins may be used in combination with other chemicals or enzymes to improve various processes. For example, a major limitation in ethanol production from biomass is the degradation of cellulose. Expansins in concert with cellulose may act synergistically in the breakdown of cellulose. If expansins help celluloses gain access to glucan chains that make up microfibrils, then they could speed cellulose action. Also, delignification is a major problem in industrial uses of many plant fibers. To solve this concern, expansins may be used with lignases (peroxidases) for synergistically de-lignifying plant fibers. Expansins may also be useful in various bioremediation applications, either above or in combination with other biological or chemical materials.

Expansins have been found in cotton fibers and are probably found in native cell walls, but are likely not to be present in processed cotton fiber walls. Accordingly, expansins may be useful to the textile industry by virtue of alteration of either the wet or dry mechanical properties of cotton flax, or other natural fibers.

It is known that certain fungal cell walls (i.e., Phycomyces) bear chemical resemblance to the structure of plant cell walls. Also, certain insects have chitin structures or walls (i.e., Manduca cuticle) that resemble fungal walls in some of their biochemistry. Expansins may modify chitin properties. Accordingly, expansins may be useful for insect control or as anti-fungal agents, either alone or in combination with other insecticidal or fungicidal agents.

A further use of expansins could be for altering the mechanical strength of gels or otherwise affecting the gelling or other properties-of gels (i.e., gelatin, gellum-gum/phytagel, agar, agarose, etc.). Such materials are useful agents in foods, cosmetics, and other similar materials. Since hydrogen bonding is important for such gels and in view of the belief that expansins alter hydrogen bonding of-wall glycons, expansins may alter the gelling properties of various gels.

An additional use of expansins may involve alteration of aggregation of hemicellulose and solubilized cellulose. In the event that expansins appropriately

affect such aggregation, they may prove useful for industrial processes involving these materials, including cellulose processing and film making.

Isolation of a novel protein allows one to attempt the cloning of the gene coding for this protein using the standard approach of establishing an amino acid sequence of a fragment of the protein and designing oligonucleotides to screen the cDNA library. When cloned, the gene for one or more expansins will need to be expressed in a bacterial or other system to obtain sufficient quantities for the commercial usefulness of the ideas listed above. Cloning will also be a necessary first step for the commercial uses requiring genetic manipulation of the protein in transgenic plants.

Engineering of plant cell growth for agricultural uses can become reality. We have evidence that expansins are important for endogenous growth of plant cells. One can envision several commercial applications of expansins. One such application includes increase of the growth of specific organs and tissues by selective enhanced expression of expansins daring plant development. Control of selective expression would be by any means such as application of specific chemicals that promote transcription of expansins or by insertion into the genome of plants specific artificial genetic constructs (i.e. appropriate promoters, enhancers, structural genes and associated elements) to effect organ-specific, tissue-specific, and/or chemical-specific enhancement of expression of the introduced or endogenous genes for the expansin proteins. Examples of use might include enhancement of fruit production (grapes, citrus fruits, etc.), enhancement of leaf growth (lettuce, spinach, cabbage), enhancement of stem or petiole growth (sugarcane, celery, flower stalks), and enhancement of root growth. Tissues engineered to grow by expression of expansins might have enhanced desirable traits (size, succulence, texture, durability).

Another direction of controlled growth would include decrease of the growth of specific organs and tissue by reducing the expression or effectiveness of endogenous expansins. The control of expression would be by any means such as application of specific chemicals that reduce transcription of expansins or by insertion of antisense genetic constructs that reduce mRNA levels of endogenous

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expansin genes, or by manipulation of the genetic control elements that regulate expression of endogenous expansin genes. Examples of such use might include dwarfing of stems for enhanced mechanical stability and genetic pruning, stunting, or elimination of undesirable plant organs.

5   Similar approaches could be utilized to control the cell size in plant cell tissue or cell cultures used in bioreactors or for production of useful chemical agents. Control might be effected by addition of exogenous expansins or inhibitors of expansin action, by genetic regulation of endogenous expansin genes and their products, or by regulation of artificially inserted genes for expansin proteins, for

10   antisense constructs against endogenous expansin mRNAs, or for proteins that regulate or modify expansin action.

Thus, while we have illustrated and described the preferred embodiment of our invention, it is to be understood that this invention is capable of variation and modification, and we, therefore, do not wish or intend to be limited to the precise

15   terms set forth, but desire and intend to avail ourselves of such changes and alterations which may be made for adapting the invention of the present invention to various usages and conditions. Accordingly, such changes and alterations are properly intended to be within the full range of equivalents and, therefore, within the purview of the following claims. The terms and expressions which have been

20   employed in the foregoing specification are used therein as terms of description and not of limitation, and thus there is no intention in the use of such terms and expressions of excluding equivalents of features shown and described or portions thereof, it being recognized that the scope of the invention is defined and limited only by the claims which follow.

25   Thus is described our invention and the manner and process of making and using it in such full, clear, concise, and exact terms so as to enable any person skilled in the art to which it pertains,-or with which it is most nearly connected to make and use the same.